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The mechanistic link between Arc/Arg3.1 expression and AMPA receptor endocytosis

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Highlights

Arc/Arg3.1 plays a key role in controlling synaptic strength and AMPA receptor endocytosis

Interactions between Arc and the CME machinery place Arc as a decisive regulator of AMPAR trafficking

The Arc-CME pathway targets specific GluA subunits for endocytosis as identified by a number of experimental approaches including rectification of AMPAR currents.

Disruption of the Arc-CME pathway in neurological diseases and as a potential therapeutic target to normalise AMPAR expression

Abstract

The activity-regulated cytoskeleton associated protein (Arc/Arg3.1) plays a key role in determining synaptic strength through facilitation of AMPA receptor (AMPAR) endocytosis. Although there is considerable data on the mechanism by which Arc induction controls synaptic plasticity and learning behaviours, several key mechanistic questions remain. Here we review data on the link between Arc expression and the clathrin-mediated endocytic pathway which internalises AMPARs and discuss the significance of Arc binding to the clathrin adaptor protein 2 (AP-2) and to endophilin/dynamin. We consider which AMPAR subunits are selected for Arc-mediated internalisation, implications for synaptic function and consider Arc as a therapeutic target.

Keywords

AMPA receptors, Arc/Arg3.1, adaptor protein 2, endocytosis, rectification

Introduction

Fast glutamatergic synaptic transmission, through the activation of ionotropic AMPARs, is one of the major mechanisms of neuronal communication in the mammalian brain. AMPARs located within the postsynaptic membrane are tetrameric in structure and consist of a homomeric or heteromeric combination of 4 known subunits (GluA1-4). The mRNA coding for the GluA2 subunit undergoes post-transcriptional editing, with a single amino-acid changed from glutamine (Q) to arginine (R). This is called Q/R editing, with AMPAR containing GluA2(Q) permeable to calcium whilst GluA2(R) containing receptors impermeable. The great majority of the GluA2 subunits expressed in the central nervous system is in the GluA2(R) form and in mature pyramidal neurons of the hippocampus a significant proportion of AMPAR present at the synapses are composed of GluA1 and GluA2 subunit heterodimers. Therefore it is probable that a large number of calcium impermeable AMPARs are present at the cell surface. The subunit composition of AMPAR not only determines Ca^{2+} permeability but also determines kinetics, rectification and receptor trafficking dynamics thus precisely tuning receptor properties to specific synaptic requirements (recently reviewed in [1,2]).

The trafficking of AMPARs in and out of the synaptic membrane is a highly dynamic process which is regulated during development, during synaptic plasticity and can be impaired during disease processes [3,4]. Although there are several mechanisms underlying the trafficking of AMPARs, one of the most studied involves the immediate gene product Arc/Arg3.1 coupling synaptic activity to the endocytosis of AMPARs. Following neural activity (such as high frequency stimulation or seizure activity) or exposure to BDNF, Arc/Arg3.1 mRNA is rapidly trafficked to postsynaptic dendritic sites and then translated leading to AMPA receptor endocytosis [5-8]. Arc is involved in specific forms of synaptic plasticity which include homeostatic scaling and LTP (discussed in other reviews in this special issue) and Arc expression is induced by group 1 metabotropic glutamate receptor (mGluR) activation resulting in AMPA receptor endocytosis leading to long term depression (mGluR-LTD) [7]. The effects of inducing mGluR-dependent LTD can be mimicked by overexpression of Arc protein in neurons which reduces the surface expression of specific GluA subunits [7]. Deletion of Arc or inhibition of its synthesis prevents AMPA receptor endocytosis, increases surface AMPARs expression and blocks mGluR-LTD. The importance of Arc in synaptic plasticity has been illustrated by reducing Arc expression in rodents, resulting in disturbances in cognitive function including impaired memory consolidation [9-12].

Although there has been extensive progress in understanding the molecular mechanisms underlying the actions of Arc and its role in synaptic plasticity, many questions still remain unanswered. In this review we will discuss three related questions:

1) How is Arc linked to the endocytic machinery that internalises AMPARs?
2) Does Arc selectively target specific subunits of AMPAR for endocytosis?
3) Arc as a potential target to manipulate AMPAR trafficking defects in disease states?

1. What links Arc expression to the internalisation of AMPARs during synaptic plasticity?

Over the last decade there have been major advances in mapping the mechanism by which activity-dependent activation of Arc expression regulates AMPAR trafficking [10,13]. It is well established that the internalisation of AMPARs from synapses is mediated by the clathrin-mediated endocytic pathway (CME) [14-18]. The first evidence that Arc-mediated

internalisation of AMPARs occurs through the CME pathway came from experiments showing that Arc interacts with endophillin and dynamin [19].

Endophilin and dynamin are accessory proteins of the CME machinery that are required for membrane constriction and scission of the clathrin-coated vesicle containing the cargo that is to be internalised (in this case AMPARs). Neither endophilin nor dynamin appear to participate in the cargo selection process as they are only involved in late phases within the sequential events of CME. Dynamin is recruited at late stages of endocytosis and its enrichment coincides with neck fission and release of the vesicle [20, 21]. Supporting the idea that endophilin is only recruited at late stages of the endocytosis process is the observation that the assembly and maturation of clathrin-coated pit formation still occurs in cortical neurons obtained from mice where distinct endophilins have been deleted [22]. These findings demonstrate that assembly and early maturation events are independent of endophilin. Therefore an interaction between Arc with either endophillin or dynamin may enhance the processes of vesicle budding and the scission of the vesicle neck but it does not place the Arc-endophillin/dynamin interaction as decisive in the selection and targeting of AMPARs for internalisation.

Recently, da Silva et al. (2016) [23] used specific anti-Arc antibodies to immunoprecipitate endogenous Arc protein from hippocampal homogenised lysate to identify novel Arc binding partners. Using this approach they identified different subunits of the adaptor protein complex-2 (AP-2) as endogenous binding partners of Arc, including the two α adaptin isoforms: α also known as αA and α2, also known as αC as well as the β2 and μ2 isoforms [23]. In contrast to the late role of dynamin and endophilin in mediating clathrin-mediated endocytosis, the AP-2 complex plays a critical role in initiating this process, as it coordinates cargo recruitment and selection together with clathrin recruitment to the plasma membrane (Figure 1,[24-27]).

To demonstrate that the Arc/AP-2 interaction is required for the endocytosis of AMPARs, da Silva et al (2016) [23] expressed in hippocampal neurons an Arc mutant construct, which cannot bind to the AP-2 complex, and showed that this blocks the Arc-dependent reduction in the AMPAR-mediated miniature excitatory postsynaptic current (mEPSC) amplitude [23,28]. This Arc-mediated effect is specific to AMPAR subunits as the internalization of EGF receptors is unaffected by expression of either Arc-WT or Arc mutant that do not bind to AP-2 [23]. Furthermore, da Silva et al. showed that expression of Arc-WT in hippocampal neurons reduces the proportion of synaptic AMPARs that lack the GluA2 subunit, an effect that is impaired in neurons expressing the Arc mutant that cannot bind the AP-2 complex [Figure 2; 23]. This finding was unexpected as the AP-2 complex has been shown to directly interact with high affinity to the GluA2 subunit but only weakly with GluA1 subunits [29]. However it is possible that other proteins interact with GluA1 subunits and then interact with the AP2-complex. An example of such a possible candidate protein is Huntingtin interacting protein 1 (HIP1) which is a component of clathrin-coated vesicles and co-localises with AP-2 [30]. In neurons obtained from HIP1$^{-/-}$ mice there is a profound defect in clathrin-mediated internalization of GluA1-containing AMPARs [30]. The observation that Arc directly binds to AP-2 provides an additional step to elucidate the mechanistic link between activity-dependent expression of Arc and the endocytosis of AMPAR subunits (Figure 1).

Thus current evidence indicates that Arc interacts with both AP-2 and with dynamin/endophillin. The role of these Arc interactions in coordinating GluA1 internalisation and their sequence within the CME pathway requires discussion. In particular, are both of these interactions or either of them alone required for Arc-mediated endocytosis? Firstly it is important to note that AP-2 and dynamin bind Arc at the same aa sequence motif, whereas endophilin binding site is situated in an independent location, which further complicates interpretation of experimental data. Thus the Arc mutant construct that does not bind AP-2

will also be unable to bind dynamin. To address this da Silva et al., [23] designed short hairpin RNA sequences to knockdown endogenous AP-2 in hippocampal cultures. In AP-2 depleted neurons, overexpression of Arc-WT failed to internalise AMPARs. To further demonstrate that Arc-AP-2 is required for AMPAR endocytosis, the authors expressed in the same lentivirus vector Arc-WT and short hairpin RNA to knockdown endogenous AP-2-emGFP-tagged, and used another lentivirus vector to express AP-2-mCherry-tagged. These two constructs were then co-expressed in the hippocampal neurons to address whether re-introduction of AP-2 in a depleted background would rescue the Arc-mediated endocytosis of AMPAR. Indeed, re-insertion of AP-2 was sufficient to rescue the Arc-WT mediated endocytosis of AMPAR, an effect that was blocked in cells expressing an Arc-mutant that cannot bind to AP-2. In these experiments endogenous dynamin and endophilin are present and were clearly insufficient to promote the internalization of AMPAR in the absence of AP-2. A model integrating the temporal sequence of clathrin-mediated endocytosis events may explain these findings. It is reasonable to propose that upon an increase in neural activity, newly expressed Arc first binds AP-2 to initiate the endocytosis process by simultaneously recruiting clathrin to the plasma membrane and by selecting the cargo (AMPAR) to be internalised. Once this process is underway the affinity between Arc and AP-2 reduces leading to the dissociation between Arc and AP-2. This makes the Arc binding domain available to dynamin. Arc-dynamin binding would then initiate/enhance dynamin polymerization and scission of the vesicle neck containing the AMPARs (see Figure 1). This proposed model is supported by the observation that dynamin polymerization, its GTPase activity and dynamin assembled stability are all enhanced in presence of Arc using in vitro assays [31].

The Arc interaction motif that binds to endophilin is not in close proximity to the interacting motifs for AP-2 and dynamin and thus it should be available to binding Arc at any stage of the CME process. Functional experiments have demonstrated that internalisation of GluA1 is significantly reduced in the absence of AP-2 even though endophilin is still present [23]. Conversely, when the interaction between Arc and endophilin is absent and the Arc-AP-2 interaction is intact endocytosis of GluA1 is inhibited [19]. The simplest explanation for these observations is that both interactions are required to internalise GluA1 with the Arc-endophilin interaction occurring downstream to the Arc-AP-2 interaction. Other possible explanations are that: a) Arc-mediates a clathrin-independent, but endophilin dependent form of endocytosis and b) the truncated form of Arc, where 10 amino acids are deleted at its N-terminal (to block endophilin binding [19]), compromise its abilities to bind to other partners and therefore non-specifically disrupts AMPAR endocytosis.

Taken together these observations suggest that the Arc interaction with AP-2 is required for targeting GluA1 containing receptors to the CME pathway and that dynamin and potentially endophilin play a critical role in coordinating the efficiency by which synaptic GluA1 containing AMPARs are internalised. However more investigation is required to clarify the precise mechanism by which Arc coordinates AMPAR endocytosis.

2. Does Arc target specific AMPA receptor subunits to endocytosis?

The findings that Arc controls excitatory synaptic transmission by decisively selecting AMPA receptors to be internalised raises an important question: which of the GluA subunits are targeted for internalisation by Arc-mediated endocytosis? Is there any specificity or can any of the four subunits (GluA1-4) be internalised? This is an important question as the targeting of specific receptor subunits for internalisation could alter the balance of the remaining synaptic receptors, changing synaptic dynamics, Ca^{2+} permeability and plasticity. Before considering this question, it is necessary to firstly define the subunit composition of synaptic AMPARs. In the adult hippocampus, there are two major forms of synaptic AMPARs: heteromers of GluA1 and GluA2 and heteromers of GluA2 and GluA3 with a small proportion of GluA1 homomeric receptors (less than 10 % [32]). The relative proportions of the heteromeric receptors is still open to debate with some studies suggesting they are of

roughly equal abundance while a recent single cell deletion study suggested 80% of receptors contain GluA1 and 2 subunits (reviewed in Henley and Wilkinson, 2016 [1]). At immature synapses the expression of GluA2 is low and increases rapidly during the first postnatal week [33, 34]. Because of the difficulty in studying Arc-mediated endocytosis of AMPAR in vivo and in acute slices, many studies have used primary hippocampal neuronal cultures instead, as the neurons are easy to transfect and manipulate. Here the composition of synaptic AMPARs is dependent on the age of the pups used to prepare the cultures (embryonic versus postnatal) and how long the cells have been cultured before they are used. For example, Pickard et al (2000) [35] showed that in primary hippocampal cultures (from 3-5 day old rat pups) only 67% of GluA1–4-positive puncta contain GluA2 immunoreactivity after 3-5 days in cultures. This ratio increases with time in culture, so that after 14 days, co-localization was almost complete with ~ 96% of GluA1-4 puncta containing GluA2 [35]. However, the precise subunit composition of synaptic receptors in neuronal culture still remains unclear (i.e. the proportion of heteromeric/homomeric receptors). Another model system used to study the mechanism by which Arc regulates synaptic AMPARs is the organotypic hippocampal slice, which has a more complete neuronal architecture, compared to neuronal cultures, and can be transfected unlike acute slices. The subunit composition of synaptic AMPARs in organotypic slices is unclear, although receptors show little rectification (see section below) consistent with few GluA2 lacking receptors [36,37]. Potential differences in AMPAR subunit composition between adult brain (and acute slices), primary cultures and organotypic slice culture can potentially complicate the interpretation of data on which of the GluA subunits are selected for internalisation following induction of Arc expression (see below).

A number of different experimental techniques have been used to determine the selectivity of Arc-endocytosis for specific GluA subunits. Surface and/or internalised AMPARs can be biotinylated and then blotted against antibodies specific for the various GluA subunits (Figure 2 A,B). This allows measurement of the ratio of internalised to surface AMPA receptor subunits. Surface AMPARs can also be directly labelled using specific antibodies targeted to the extracellular N-terminus sequences. The major disadvantage of these labelling techniques is a lack of specificity for synaptic receptors as extra-synaptic receptors will also be labelled.

A second approach is to use electrophysiological recordings to measure the degree of AMPAR rectification, AMPAR are isolated by blocking both NMDA and $GABA_A$ receptors. Here the amplitude of either AMPAR-mediated miniature excitatory post synaptic currents (EPSCs) or evoked EPSCs is measured at two different holding potentials (usually -60 and + 40 mV, illustrated in Figure 2C-E). The amount of current passed through the AMPARs at the different holding currents depends on receptor subunit composition. If the AMPARs lack GluA2 subunits, then they can be blocked in a voltage-dependent manner by a class of molecules called polyamines (spermine is normally added to the patch pipette solution). When the neuron is at a depolarized membrane potential (+ 40 mV), the polyamines block the AMPAR channel more strongly, preventing the passage of ions through the channel. Thus GluA2-lacking AMPARs are said to be inwardly rectifying which means that they pass less outward current than inward current ([38-42]; Figure 2C,D). In contrast, receptors containing edited GluA2 subunits have a linear current-voltage relationship [43, 44]. The amount of rectification is quantified by the rectification index which is the ratio of the AMPAR-mediated current amplitude at the depolarised potential relative to the current amplitude at the hyperpolarised potential (Figure 2E). The rectification index is a little counter-intuitive as an increase in the rectification index means there is less rectification. In the adult hippocampus, CA1-CA3 synapses have a high rectification index, as most receptors contain edited GluA2 [45]. In hippocampal cultures, the rectification index maybe lower as the GluA2 subunit may not be expressed at all synapses depending on the age of the culture [35].

To determine whether Arc preferentially targets specific AMPAR subunits for endocytosis both AMPA receptor surface labelling and electrophysiological recordings have been used in

combination with either Arc overexpression or knockdown and the use of a chemical protocol to induce Arc protein expression. It is well-established that exposure of the group I metabotropic receptor agonist (DHPG) induces mGluR-LTD in cultured hippocampal neurons and in acute hippocampal slices [46,47]. This form of synaptic plasticity is Arc-dependent [7].

There is substantial evidence, that in primary neuronal cultures, Arc mediates the internalisation of GluA1 subunits. For example, Chowdhury et al (2006) [19], Shepherd et al (2006) [28] and Waung et al (2008) [7] all showed that changes in Arc expression could either reduce the surface expression of GluA1 subunits (with Arc overexpression) or increase GluA1 surface expression (with Arc knockdown). Waung et al (2008) [7] additionally showed that Arc overexpression occludes the mGluR-induced (DHPG) decrease in surface GluA1 expression. In an interesting study, Peebles et al (2010) [48] showed that Arc not only mediates GluA1 endocytosis but also regulates changes in spine morphology. Two further studies [49, 50] have modified Arc expression indirectly, by targeting the enzymes that ubiquitinate Arc, and have showed changes in surface GluA1 subunit expression consistent with the alterations in Arc metabolism and protein expression.

Taken together this data provides strong evidence that GluA1 is internalised via Arc-mediated endocytosis in neuronal cultures. As previously mentioned, at mature hippocampal synapses, most GluA1 is combined with GluA2 in heteromeric receptors with little GluA1 present in the form of homomeric receptors [32]. Thus it would be expected that GluA2 would also be internalised by Arc. However, contrary to expectations, there is little or no accompanied change in the expression of GluA2 subunits in neuronal cultures. For example, Shepherd et al (2006) [28] reported that surface levels of GluA2 remain unchanged even though GluA1 surface expression is significantly decreased by Arc overexpression. Eales et al (2014)[47] showed that application of DHPG to hippocampal cultures induces endocytosis of GluA1 subunits, increases the rectification index of mEPSC amplitudes but does not internalise GluA2 subunits. More recently da Silva et al (2016) [23] showed that Arc overexpression facilitates the internalisation of expressed GluA1 subunits but not GluA2 subunits in H4 neuroglioma cell lines (Figure 2 A,B). Furthermore they showed in primary hippocampal neuronal cultures that overexpression of Arc increased the rectification index which is consistent with a preferential loss of GluA1 containing receptors [23; Figure 2F,G].

If GluA1 is internalised by Arc-mediated endocytosis, why do GluA2 surface levels remain unchanged? The increase in rectification index [23,47] is consistent with a preferential loss of GluA1 homomeric receptors leaving a higher proportion of AMPA receptors containing GluA2 at synapses. Thus the simplest explanation for these observations is that neuronal cultures express a higher proportion of GluA1 homomeric receptors compared to the amount found in the adult hippocampus. A recent study by Takemoto et al (2017) [51] supports this proposal. They showed that optically inactivating GluA1 or by applying an agent (NASPM) which blocks AMPARs lacking the GluA2 subunit reduced the AMPAR-mediated synaptic currents to ~50% in hippocampal primary cultures, indicating that approximately half of the AMPAR-mediated synaptic current was mediated by GluA1 homomeric receptors. They suggest that high levels of spontaneous activity in primary cultures can drive GluA1 into synapses increasing the level of synaptic GluA1 homomeric receptors [51,52]. Because the single-channel conductance is about four times greater in GluA2-lacking AMPARs than in GluA2-containing ones [53], even the removal of a small fraction of GluA1 homomeric receptors at synapses via Arc-facilitated endocytosis could be responsible for a relatively large reduction in the synaptic AMPAR-mediated current.

Rial Verde et al (2006) [54] investigated the actions of Arc in organotypic hippocampal slice cultures (prepared from 5-7 day old rats and cultured for 6-9 days). They reported that, in contrast to neuronal culture studies, AMPAR composed by GluA2/3 subunits are preferentially targeted for internalisation by Arc. They first showed that when Arc was over-expressed (using Sindbis virus) AMPAR-mediated evoked EPSCs and miniature EPSCs

were reduced in amplitude. They then used two approaches, firstly they expressed the GluA2 c-terminus tail peptide which prevents the synaptic delivery of GluA2/3 receptors and also reduces basal synaptic transmission. When Arc was co-expressed with the GluA2-c tail peptide there was no further depression in surface receptor expression consistent with Arc preferentially internalising GluA2 subunits [54]. They also co-expressed Arc with pepAP-2, a peptide which prevents the interaction between GluA2 and the AP-2 complex. This abolished the Arc mediated reduction in AMPAR-mediated currents [54]. Finally they performed biotinylation experiments in hippocampal cultured slices overexpressing Arc to show no change in surface GluA1 expression but a reduction in GluA2 surface expression levels [54].

Why are there such striking differences in the results produced using organotypic slice cultures and dissociated neuronal cultures? This is currently unclear. It has been suggested that differences in the speed of receptor recycling between cultured neurons versus organotypic slice cultures may change the selectivity of Arc for GluA subunits [54]. It has been proposed that in organotypic hippocampal slices the recycling of GluA1/2 receptors is slow compared to GluA2/3 containing receptors. Thus GluA2/3 receptors are preferentially internalised. In contrast the recycling of GluA1/2 is much faster in primary neuronal cultures. If this is correct then Arc selectivity is determined by the availability of the receptor pool for recycling [54].

However, the main unanswered question is which molecules mediate Arc selection of AMPAR subunits to endocytosis *in vivo.*

Data from neuronal cultures and organotypic hippocampal slices suggest that either GluA1 or GluA2 can be endocytosed by Arc. This suggests that there is nothing specific to the subunit sequence but instead targeting may instead just depend on subunit availability [54]. However this does not fit with the data from da Silva et al. (2016) [23] who showed that GluA2 subunits were not internalised with Arc co-expression although GluA1 subunits were. It could be argued that organotypic slices provide a more accurate representation of the in vivo brain than neuronal cultures, since neuronal architecture is more complete. However the lack of afferent connections to neurons within cultured slices, combined with the loss of efferent connectivity to areas outside the slice elicits a reorganization and expansion of intrinsic axons [55]. This over connectivity and lack of activity could lead to changes in synaptic AMPAR properties. To fully define the selectivity of Arc that actually occurs in the brain, there is a requirement for the imaging of synaptic receptors in awake behaving animals. Due to technical limitations this is currently not possible. However in recent years there have been substantial technical developments which increase the resolution in the visualisation of synaptic AMPAR subunit trafficking. Therefore, to show whether Arc targets specific AMPAR subunits for endocytosis, fluorescent tagged Arc could be overexpressed using neuron-specific lentiviral vectors in vivo and then combined with fluorescent chemical compounds to label endogenous surface AMPAR subunits in acute slices [56, 57]. This approach combined with high-performance/resolution live imaging techniques [58, 59] would allow addressing whether Arc selectively targets specific AMPAR subunits to be internalised. This approach could also be used in combination with measurement of rectification of AMPAR-mediated mEPSCs amplitudes in acutely isolated slices (with Arc overexpressing neurons identified by fluorescence). These approaches would determine whether or not Arc selectively targets specific AMPAR subunits for endocytosis.

3. The Arc-dependent endocytic pathway as a potential therapeutic target

Many neurological and neurodegenerative diseases including autism [60], Rett syndrome [61], schizophrenia [62] depression [63], epilepsy [64-66] and Alzheimer's disease [67] are associated with deficits in synaptic transmission and in particular, abnormalities in the trafficking of synaptic AMPAR. Defects in Arc function may underlie some of the observed changes in synaptic AMPAR trafficking and expression. For example, Arc probably plays a

role in the synaptic changes that occur in epilepsy, as Arc is strongly induced by seizure activity [68] (and that is how it was originally discovered [69]). It is tempting to link the many reports of increased forgetfulness in epileptic patients [70, 71] with enhanced AMPA receptor endocytosis in the hippocampus that presumably follow seizure-induced Arc expression, although there is currently no direct evidence for such a link. Disturbances in Arc induction may also play a role in the development of a range of neuropsychiatric disorders through changes in the transcription factors that activate the *Arc* gene. For example, synaptic activity induces the activation of the *M*yocyte *E*nhancer *F*actor 2 (MEF2) family of transcription factors resulting in the activation of a number of genes including Arc and the selective elimination of glutamatergic synapses onto hippocampal and striatal neurons [72-76]. The RNA binding protein, Fragile X Mental Retardation Protein (FMRP) is required for MEF2-triggered synapse elimination [74, 76] along with the activation of metabotropic glutamate receptor 5 (mGluR5) on the dendrites of hippocampal CA1 neurons. Loss of function mutations in FMR1, MEF2c are linked with intellectual disability, autism and/or schizophrenia [77, 78]. Deficits in MEF2-induced synapse elimination that occur in Fragile X Syndrome models are likely to lead to the observed increase in dendritic spine density and deficits in experience-dependent spine elimination onto cortical neurons [79, 80]. Abnormal removal of Arc protein appears to occur in Angelman syndrome, a genetic disorder producing severe physical and intellectual disability. In a mouse model of Angelman syndrome there is enhanced AMPAR endocytosis leading to a decrease in hippocampal synaptic AMPARs [49]. The excessive internalization of AMPARs is likely to result from the failure to ubiquitinate and degrade Arc, due to the deficiency of Ube3A (an ubiquitin-protein ligase). Other Ube3A substrates, in addition to Arc, also probably contribute to the development of neurological disturbances. In Alzheimer's disease (AD) patients it has been reported that Arc expression levels are significantly increased in the prefrontal cortex [67]. In a mouse model of AD (APP/PS1) there is a disruption in patterned Arc expression with expression reduced in some neurons but greatly overexpressed in other neurons [81]. These effects would be predicted to lead to abnormal levels of endocytosis of AMPAR subunits and these have indeed been reported [67, 82-84]. However, the precise GluA subunits that are internalised in these AD models remain contentious.

A possible therapeutic intervention for diseases where AMPAR expression is abnormal is the manipulation of the Arc-CME pathway, in an attempt to normalise AMPAR subunit expression. The key position of Arc in the control of both synaptic strength and the composition of synaptic receptors together with its restricted expression (in neurons) makes it a strong candidate for such manipulation. In contrast, the ubiquitous nature of the CME pathway makes it a less favourable target; with for example mutations in endophilin and dynamin leading to disruptions in synaptic transmission [85, 86] and AP-2 null mutations in mice are lethal [87]. It is possible that additional proteins that have yet to be identified link Arc to the CME pathway and the internalisation of AMPAR subunits and these proteins may be useful therapeutic targets. It is also important to identify which AMPAR subunits are internalised by Arc, as this will determine the changes in synaptic receptor compositions produced by Arc manipulation. Thus it is important to fully define the Arc-CME pathway and to determine which AMPAR subunits are internalised through Arc induction. This research highlights the importance of basic science to fully understanding signalling pathways in healthy conditions which then identifies targets for therapeutic intervention in pathological states.

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Figure Legends

Fig 1. Arc binds to AP-2 and endophilin/dynamin to facilitate AMPAR endocytosis.

A proposed model of the mechanism by which Arc interacts with the clathrin mediated endocytosis (CME) pathway to facilitate AMPAR endocytosis. An increase in neuronal activity (or exposure to BDNF) promotes rapid Arc mRNA translation and protein expression at the dendritic spines. (1) Newly expressed Arc binds to the AP-2 complex, which binds directly or indirectly to AMPAR subunits at the plasma membrane. The identity of these GluA subunits is still open to debate and is discussed in details in the review. To initiate the formation of the clathrin-coated assembly AP-2 also binds and recruits clathrin to the membrane. Once formation of the pit is initiated, the binding affinity between Arc and AP-2 is reduced and Arc dissociates from AP-2. (3) Arc is then available to bind and recruit dynamin and endophilin to promote scission of the endocytic vesicle containing the AMPAR to then be targeted for either recycling or degradation (4). Please note that AP-2 and dynamin bind to Arc at the same motif [19, 23], therefore it is likely that Arc first binds to AP-2 and then binds to dynamin. Endophilin binds Arc at a different motif which allows it to bind to Arc at any time during the CME temporal sequence of events. Although this model fits the available data, additional experiments are required to either confirm or refute it.

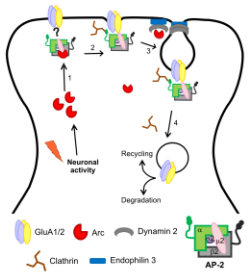

Figure 1

Fig 2. Arc-facilitates endocytosis of GluA1 subunits and increases the rectification index.

(A,B) Representative blots showing that $Arc_{(WT)}$, but not the $Arc_{(W197A)}$ mutant (that is unable bind to AP-2), facilitates GluA1, but not GluA2 endocytosis in H4 neuroglioma cells expressing myc-GluA1 (A) or myc-GluA2 (B) in combination with either: empty pCIneo vector, pCIneo $Arc_{(WT)}$ or pCIneo $Arc_{(W197A)}$. Western blot band densitometry analysis showing that: (a) $Arc_{(WT)}$, but not $Arc_{(W197A)}$, promotes a significant reduction in surface expression of GluA1 subunits. However expression of either $Arc_{(WT)}$ or $Arc_{(W197A)}$ does not promote any changes in surface expression of GluA2 subunits(from daSilva et al., 2016; [23]). (C-E) Schematic panels constructed to illustrate the difference in rectification properties between AMPAR containing GluA2 subunits (blue) and those lacking GluA2 subunits (red). (C) Current-voltage relationship for AMPAR-mediated currents, relative to the AMPAR-mediated current recorded at -60 mV and at a range of holding potentials from -60 mV to +40 mV. Receptors which contain GluA2 have a linear current-voltage relationship whereas receptors lacking GluA2 show rectification when the cell is hold at positive potentials. (D) AMPAR-mediated mEPSC waveforms recorded at -60 mV and at + 40 mV for AMPAR containing GluA2 subunits (blue) and in those lacking GluA2 subunits (red). The mEPSCs produced by AMPAR lacking GluA2 subunits show rectification (smaller amplitude of mEPSCs at positive holding potentials). (E) Graph plotting the rectification index (amplitude at +40 mV/ amplitude at -60 mV) for the mEPSCs in (D). The rectification index for mEPSCs produced by AMPAR lacking GluA2 is smaller (shows more rectification) than for mEPSCs produced by AMPAR containing GluA2. (F) Representative average mEPSC waveforms recorded at a holding potential of -60 and + 40 mV for cells expressing GFP, $Arc_{(WT)}$ and $Arc_{(W197A)}$ in the presence of spermine (100 µM) in the intracellular solution. (G) Bar chart plotting the mean rectification index (peak amplitude at + 40 mV divided by peak amplitude at -60 mV) for neurons expressing GFP, $Arc_{(WT)}$ and $Arc_{(W197A)}$;. Note that $Arc_{(WT)}$ reduces the amount of rectification (as seen as an increase in the rectification index) whereas $Arc_{(W197A)}$ has significantly less effect on rectification, suggesting that Arc preferentially targets GluA1 for endocytosis (from da Silva et al., 2016; [23]).

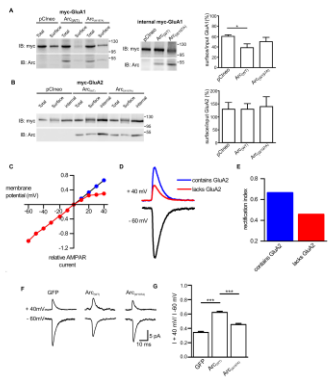

Figure 2